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Role of PAK4 in Pancreas Development and Breast Cancer

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Role of PAK4 in Pancreas Development and Breast Cancer

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To Time I spend on PhD

Abstract

PAK4 is a Rho GTPase-regulated serine/threonine kinase that possesses critical functions in embryonic, neuronal and vascular development, immune defense and cancer. Constitutive PAK4 depletion causes embryonic lethality and mice with conditional PAK4 gene depletion in the heart and in the central nervous system displayed serious organ defects. PAK4 is overexpressed and genetically amplified in cancer cell lines as well as in cancer patients, including pancreas and breast. PAK4 also regulates many cellular functions related to cancer progression, including cell proliferation, survival and cell cycle as well as cell morphology, adhesion and migration, which are dependent on the actin cytoskeleton. Therefore, better understanding of the role of PAK4 in development, cancer progression and the mechanism behind that could help us to find suitable strategies to treat cancer.

In paper I, we elucidated the role of PAK4 in pancreas development. PAK4 knockout mice were born at Mendelian ratios. Morphological and immunohistochemical examinations and quantifications indicated that exocrine, endocrine and ductal compartments retained the normal proportions and distributions upon PAK4 gene depletion. In addition, body weight records and a glucose tolerance test revealed no differences between wild type and PAK4 knockout mice. Together, this suggests that PAK4 is dispensable for mouse pancreas development. This will facilitate future use of our Pdx1-Cre-driven conditional PAK4 knock out mouse model for testing *in vivo* potential functions of PAK4 in pancreatic disease models such as for pancreatitis and different pancreatic cancer forms.

In paper II, we comprehensively characterized the human PAK4 interactome. We found that the PAK4 interactome was enriched in diverse protein networks, including the 14-3-3, proteasome, replication fork, CCT and Arp2/3 complexes. Moreover, we found that PAK4 interacts with and phosphorylates VCA domain at Ser484/Ser485 and promotes Arp2/3-dependent actin polymerization *in vitro*. Also, PAK4 ablation *in vivo* reduced N-WASP Ser484/Ser485 phosphorylation and altered the cellular balance between G- and F-actin as well as the actin organization. By presenting the PAK4 interactome, we here provide a powerful resource for further investigations and we also indicate a novel mechanism by which PAK4 may regulate actin cytoskeleton remodeling.

In paper III, we established PAK4 as a protumourigenic regulator of breast cancer acting through the non-canonical NF-κB subunit RelB. Our results demonstrated that PAK4 was overexpressed in human breast cancer and associated with poor prognosis. We found that PAK4 inhibition arrested cancer cell growth by inducing several senescence-like features. RNA sequencing and subsequent mechanistic exploration revealed that PAK4 inhibited NF-κB signaling. Further, we identified RelB, a subunit of the non-canonical NF-κB family, as necessary for senescence-like growth arrest upon PAK4 depletion. Importantly, we pinpointed RelB as a direct substrate of PAK4 and mapped a PAK4 phosphorylation residue (S151) within the Rel-homology domain that directly influences RelB-DNA interactions and target gene expression.

Taken together, these studies highlight the importance of PAK4 in cancer and provide new mechanisms and new views to understanding the role of PAK4 in cancer cell progression.

List of Scientific Papers

I. **Miao Zhao**, Parisa Rabieifar, Tânia D. F. Costa, Ting Zhuang, Audrey Minden, Matthias Löhr, Rainer Heuchel, Staffan Strömblad (2017). Pdx1-Cre-driven conditional gene depletion suggests PAK4 as dispensable for mouse pancreas development, Scientific reports, 7: 7031, doi: 10.1038/s41598-017-07322-5.

II. **Miao Zhao**, Matthias Spiess, Henrik J. Johansson, Helene Olofsson, Jianjiang Hu, Janne Lehtiö, Staffan Strömblad (2017). Identification of the PAK4 interactome reveals PAK4 phosphorylation of N-WASP and promotion of Arp2/3-dependent actin polymerization, Oncotarget, https://doi.org/10.18632/oncotarget.20352

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List of abbreviations

AID	autoinhibitory domain
Arp2/3 complex	actin-related protein 2/3 complex
CCT complex	chaperonin containing TCP-1 complex
HER2	human epidermal growth factor receptor 2
IP	immunoprecipitation
iTRAQ	isobaric tag for relative and absolute quantitation
KO	knockout
MMTV	mouse mammary tumor virus
MS	mass spectrometry
PAKs	p21-activated kinases
PBD	p21-binding domain
PDAC	pancreatic ductal adenocarcinoma
PyMT	polyomavirus middle T
ROS	reactive oxygen species
SA-β-gal	senescence associated β galactosidase activity
WT	wild type

1 Introduction

1.1 Cancer

In human body, normal cells growth, division and death are tightly controlled by different gene and microenvironment. When our body's control mechanism stops working or does not work properly, cancer will develop. Instead of death, cancer cells proliferate without control, forming more and more abnormal cells. Cancer is genetic disorder causing by sequential gene mutation accumulation[1]. Cancer is also a collection of diseases which can start almost anywhere in the human body, cancer cells divide without stopping and spread into surrounding tissues[2]. There are many possible causes inducing cancer, most common causes are smoking, radiation from sun or x-rays, alcohol, virus. Age and hereditary are also the very important cancer risk facts.

As the second leading cause of death, cancer make about 0.6 million Americans death in 2016[3]. Lung, liver, colorectal, stomach, breast, pancreas cancer are the common causes of the cancer death globally.

1.1.1 The hallmarks of cancer

1.1.1.1 The hallmarks of cancer

Different cancer may come from different parts of body, but they all share at least ten acquired distinctive and complementary capabilities[4,5] (Figure 1). 1) Sustaining proliferation signaling. In normal tissues, growth signaling is carefully controlled to ensure the tissue have a normal architecture and function. To achieve rapid cell proliferation, cancer cells may carry somatic mutations to constitutively activate the cell replication or may disrupt normal negative-feedback loops. 2) Evading growth suppressors. Cancer cells often display mutations or malfunction of tumor suppressors (e.g. p53, pRB) to avoid limitation of cell growth and proliferation. 3) Resisting cell death. Tumor cells use different ways to limit apoptosis. Loss of p53 tumor suppressor function is the most common way by which cancer cells can get rid of this damage sensor. Except for that, tumors may increase the expression of anti-apoptotic regulators or by down regulating pro-apoptotic factors to achieve protection from apoptosis. 4) Enabling replication immortality. Cancer cells can bypass senescence and apoptosis, two natural barriers to proliferation, to exhibit unlimited replicative potential. 5) Inducing angiogenesis. Like normal cells, cancer cells need vasculature to supply nutrients as well as oxygen and to evacuate metabolic wastes. In most adult tissues, angiogenesis is quiescent, with the exception of a few physiologic processes like wound healing and female reproductive cycling. However, during cancer progression, angiogenesis is always turned on, causing quiescent vasculature to grow more vessels which help cancer cells growth. 6) Activating invasion and metastasis. From invasion to metastasis is a multistep process[6], which begin from local invasion, then intravasate into surrounding blood vessels

(intravasation), next, escape from the vessel lumina into the parenchyma of distant tissues (extravasation). Cancer cells can form small lesions (micrometastases) in distant tissues, and finally these small lesions can grow into big tumors (colonization). 7) Genome instability and mutation. Although the types of genome alteration are different in different cancer forms, large numbers of genome repair system defects, gene copy number and single-nucleotide polymorphism destabilizations are common in different kinds of cancer. 8) Tumor promoting inflammation. Inflammation does not just happen in the early stages of tumorgenesis, but also appear during the entire cancer progression. Inflammatory cells can release reactive oxygen species (ROS), which can cause mutations in the DNA of nearby cancer cells, thereby accelerating cancer towards states of malignancy. 9) Deregulating normal energy metabolism. In the aerobic environment, normal cells use glucose by glycolysis in the cytosol and then to carbon dioxide in the mitochondria; while in anaerobic conditions, cells prefer to use glycolysis alone. Warburg found that cancer cells, even under aerobic environment, could reprogram their glucose metabolism to use mainly glycolysis. 10) Avoiding immune destruction. To avoid the normal immune system to eliminate cancer cells, immunogenic cancer cells may disable some components of the immune system[4,5].

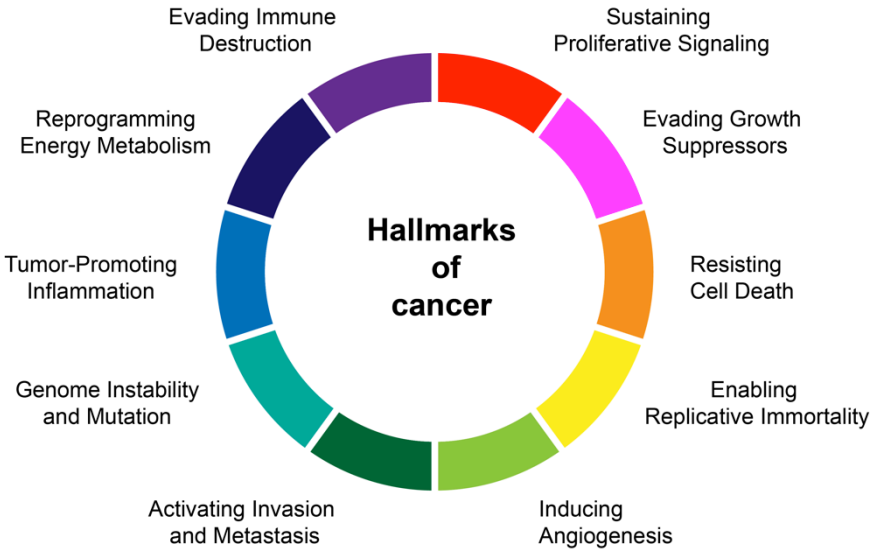

Figure 1. The hallmarks of cancer. This figure shows the ten hallmark features of the cancer as depicted by Douglas Hanahan and Robert A. Weinberg, Cell, 2011, 144, 5, 646–674.

1.1.1.2 Cellular senescence

More than 50 years ago, Leonard and Paul found that normal fibroblasts couldn't grow indefinitely in culture, a phenomenon they called "cellular senescence". Now we know that lacking telomerase activity causes this phenomenon. What is cellular senescence? As showed in the Figure 2, senescence cells include multiple of the following features: 1) flattened and enlarged morphology; 2) lacking of proliferation; 3) expression of senescence associated β galactosidase activity (SA-β-gal); 4) expression of cell cycle inhibitors; 5) displaying of

senescence associated secretory phenotype; 6) expression of genomic damage makers and displaying of senescence associated heterochromatin foci. However, no single feature is completely specific or universal for all the senescence types[7].

There are at least four different types of cellular senescence: DNA damage induced senescence, replicative senescence, stress induced senescence and oncogene induced senescence. DNA damage and telomere shortening can both trigger a DNA damage response, mediated by ATM, ATR and CHK1/2, leading to phosphorylation of p53 triggering down-stream pathways. Many different types of stress can induce ROS, e.g. ROS is induced by the RAS-RAF-MEK-ERK pathway, which can active the p38 MAPK, leading to increase p53 and p21. Oncogenes can also induce DNA damage response or ROS to further induce senescence[7,8]. Different stimulus can trigger senescence by different pathways, but many of them can activate p53, p15, p16, p21, and/or p27, inhibiting the CDK cyclin complexes and hypo-phosphorylated pRB, finally triggering proliferation arrest[7].

Senescence plays an important role in development, different diseases and during ageing, but here I will focus on senescence in cancer progression. Senescent cells are present in many premalignant lesions. In a KRAS ^{G12V} expressing mouse model, senescence is present at early stages of lung and pancreatic tumors[9,10]. BRAF^{V600E} mutant also results in an increasing number of senescent cells in lung adenomas[11,12]. Inactivation of tumor suppressors, like PTEN, also can induce senescence in preneoplastic lesions[13]. However, senescence does not only occur in premalignant lesions, but also works as an effective barrier to tumor progression. For example, p53 loss accelerates the appearance of metastatic prostate cancer in PTEN-/- prostatic intraepithelial neoplasia lesions[13]. This example suggests that if mutations or other alterations cannot disable senescence to occur, oncogene induced senescence will block further growth and a tumor will not appear. Interestingly, chemotherapeutic drugs and ionizing radiation can induce senescence in cancer cells[14]. Taken together, pro-senescence therapies could be an effective way to treat cancer. Therefore, cellular senescence inducing drugs could be a useful approach for the development of novel cancer treatment.

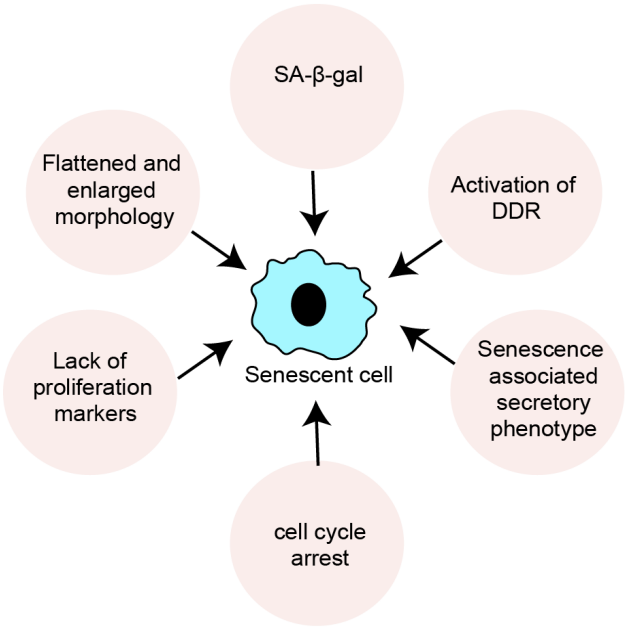

Figure 2. Biomarkers of senescence cell.

DDR: DNA damage response; SA-β-gal: senescence associated β galactosidase activity. (Based on Burton et al. Cell. Mol. Life Sci. (2014) 71: 4373)

1.1.2 Breast and breast cancer

1.1.2.1 Breast anatomy and physiology

The breast in the adult woman is a milk-producing, tear-shaped gland. The structure of breast includes glandular, fatty and fibrous tissues, as well as nipple and areola. Glands are made up by 15-20 lobules and ducts, where the lobules produce milk and ducts carry the milk to the nipple[15]. Breast development starts from puberty and undergoes dramatic changes with the cycle of pregnancy/lactation/involution[16]. In the early pregnancy, the epithelial cells start extensive proliferation; and then in lactation period, epithelial cells differentiate to be specialized cells that express different milk components. When an infant is weaning, this process causes involution, in which most of the epithelial cells quickly die and the rest of the cells becomes a glandular structure that look like the pre-pregnant state[16].

1.1.2.2 Breast cancer Epidemiology

Breast cancer is the most common cancer for women. Breast cancer causes the most women cancer deaths in the United States followed by lung cancer. Epidemiologic studies reveal a variety of risk factors for breast cancer. These risk factors include: Caucasian genetic origin; Country: development country; Age: younger age at parity and menarche, older age at first pregnancy or menopause; Family: breast cancer in the first-degree when young; Diet: red meat, animal fat and less consumption of fruits and vegetables; Hormone: hormone replacement therapy after menopause; alcohol consumption; exposure to ionizing radiation. Decreasing factors include: increasing physical activity and breast-feeding[17-20].

1.1.2.3 Breast cancer classification

Breast cancers with different histopathological and biological features reveal different behaviors; therefore accurate grouping of breast cancers into clinically relevant subtypes is very importance for assessing prognosis and determining the appropriate therapy[21,22]. These clinical and pathological factors includes: patient age, histological features, tumor size, lymph node status and hormone receptor status[23]. Combining all these factors is more valuable than just viewing each one isolated.

Gene expression profiling is used to classify breast cancer and to predict therapy response. Based on transcriptional expression patterns, breast cancer can be classified into five different groups[21,22,24]: Luminal A, Luminal B, Human epidermal growth factor receptor 2 (HER2) type, Basel-like and Normal-like. Luminal A and Luminal B are the estrogen receptors and/or progesterone receptors positive, and comprise about 75% of breast cancers. As the most common of breast cancer subtype, Luminal A represents 50%-60% of breast cancers. Luminal A cancers usually display high ER, low proliferation related genes, low histological grade, low mitotic activity, low degree of nuclear pleomorphism and carry relatively good prognosis. Luminal B comprises 15%-20% of breast cancers and is more aggressive than Luminal A. On average, Luminal B breast cancers have higher histological grade, higher proliferative index and a worse prognosis comparing to Luminal A. HER2-positive cancer

comprise about 15-20% of breast cancer. However, not all HER2-positive breast cancers belong to the HER2 molecular subtype. The HER2 breast cancer type tends to display high tumor grade, lymph node-positive and a worse prognosis than the luminal subtypes. The Basal-like subtype accounts about 8%-37% of breast cancers. Most of Basal-like breast cancers overlap with the classification as triple negative breast cancers (estrogen receptors, progesterone receptors and HER2 are all negative). The Basal-like subtype is characterized by high proliferation, high histological, nuclear grade and a poor prognosis[21,22,24]. Normal-like subtype accounts for about 5%-10% of all breast cancer, triple negative, cytokeratin5/6 and epidermal growth factor receptor negative, have good prognosis. There are a doubt about weather normal-like tumor is considered as a special subtypes or not[22].

1.1.2.4 Breast cancer treatment and challenges

Over the past half-century, the five years survival rates of breast cancer patients have significantly increased thought combining the locoregional treatment and adjuvant systemic therapies. Locoregional therapy means local treatment of the cancer that does not affect the rest of the body; this includes surgery and radiation therapy. Surgery is usually the primary therapy for breast cancer. Systemic treatments means that the treatment can reach cancer cells anywhere in the body and is given in the forms of chemotherapy, hormone therapy, and/or targeted therapy. Chemotherapy can be given before surgery (neoadjuvant chemotherapy) and also after surgery (adjuvant chemotherapy). Estrogen receptors-positive and progesterone receptors-positive breast cancer can benefit from anti-hormone therapy, while HER2-positive breast cancer can benefit from treatments targeting HER2. Breast cancer treatment have been improved, but it still causes large numbers of lethality in cancer, so it is very important to find a new strategy to treat breast cancer[25].

1.1.3 Pancreas and pancreatic cancer

1.1.3.1 Pancreatic anatomy and physiology

Pancreas is a flat organ located deep in the human belly. Pancreas is very interesting, because it intimately mixes two glands together into one organ[26]. One of these glands is called the exocrine pancreas, including mainly acinar and ductal cells. The exocrine acinar cells release enzymes into a series of ducts, finally entering the duodenum to help with food digestion. The exocrine components comprise more than 95% of the pancreatic mass. Another gland is called the endocrine pancreas. The endocrine pancreas also is called the islets of Langerhans, which is composed of small islands of cells. Islets comprise 1-2% of the pancreatic mass. The endocrine pancreas have five different cell types, α cell (glucagon), β cell (insulin), ε cell (ghrelin) δ cell (somatostatin) and PP cell (pancreatic polypeptides), which release different hormones into the bloodstream to help control blood glucose level (Figure 3).

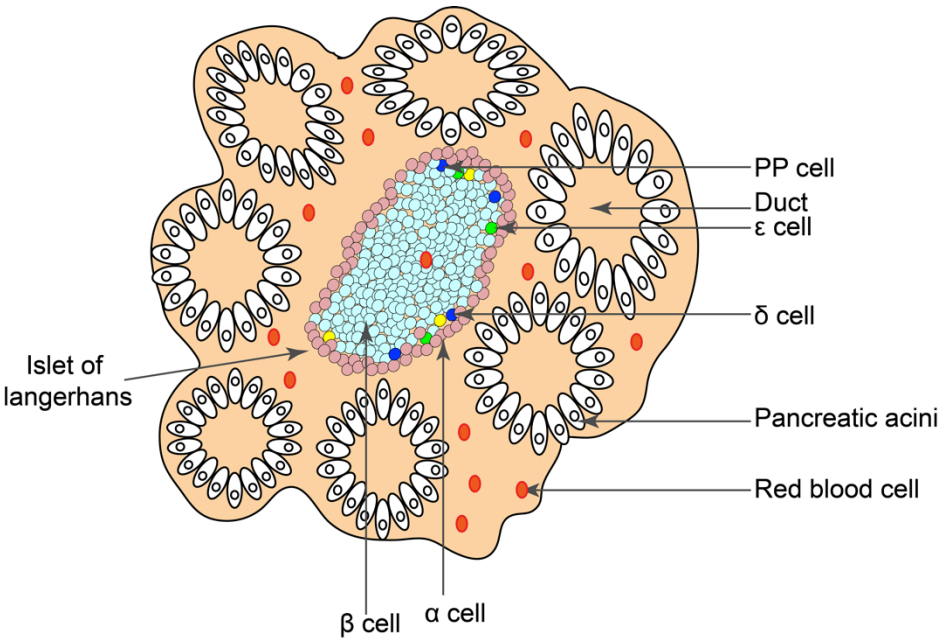

Figure 3. Cell types and organization of pancreas. This illustration shows the major cell types and their arrangement in pancreas. Pancreas includes exocrine acini and islets of Langerhans. The acini are composed of exocrine acinar cells and ductal cell mainly. The islands of Langerhans contain α, β, δ, ε and PP (pancreatic polypeptide) cells, as well as blood cells. (Based on http://www.differencebetween.info/difference-between-alpha-cell-and-beta-cell).

1.1.3.2 Pancreatic cancer

Pancreas cancer, especially pancreatic ductal adenocarcinoma (PDAC), which account for more than 95% of malignant tumors of the pancreas, carries a poor prognosis with a five-year survival of 3%–5% after discovery and a median survival of six months[27-29]. Today, some risk factors have been identified: age, smoking, obesity and diabetes, consumption of red or processed meat and high-temperature cooking, a family history of pancreatic cancer and chronic pancreatitis all have an increased risk of developing pancreatic cancer[27-29]. Targeted therapy has significant effect in the treatment for many cancers, including breast, melanoma, lung and colorectal cancer, but there is currently no targeted therapy with significant effect on pancreatic ductal adenocarcinoma. Pancreatic ductal adenocarcinoma remains a lethal disease and is estimated to be the second in the cancer related death by 2030[30,31]. Gemcitabine is the current standard treatment[27].

1.2 p21-activated kinase 4

1.2.1 Small GTPases

Three decades ago, the Ras oncogene was the first small GTPase to be discovered, till today a large number of relative proteins forming the small GTPase superfamily have been discovered [32,33]. Small GTPases are GDP-/GTP-regulated molecular switches. Generally, the GDP-binding form is inactive while the GTP-binding form is active[33]. Because GDP is tightly bound and GTP is hydrolyzed slowly, the regulating proteins guanine nucleotide

exchange factors (GEFs) accelerate GDP dissociation while GTPase activating proteins (GAPs) motivate GTP hydrolysis[32] (Figure 4).

According to sequence homologies and functional similarity, the small GTPases are divided into five subfamilies, the Ras, Rab, Arf, Ran and Rho subfamilies[34]. The Ras subfamily proteins play key roles in cell proliferation, differentiation, apoptosis, survival and gene expression[32]. The Rab subfamily proteins regulate membrane and protein trafficking in the secretory and endocytic pathways; the Arf subfamily proteins regulate endocytosis, exocytosis and vesicular trafficking; Ran regulates mitotic spindle organization and nucleus to cytoplasm transportation and the Rho subfamily proteins including Rac1, Rho and Cdc42 are involved in regulation of cytoskeletal organization, cell polarity, cell cycle and gene expression[32-35].

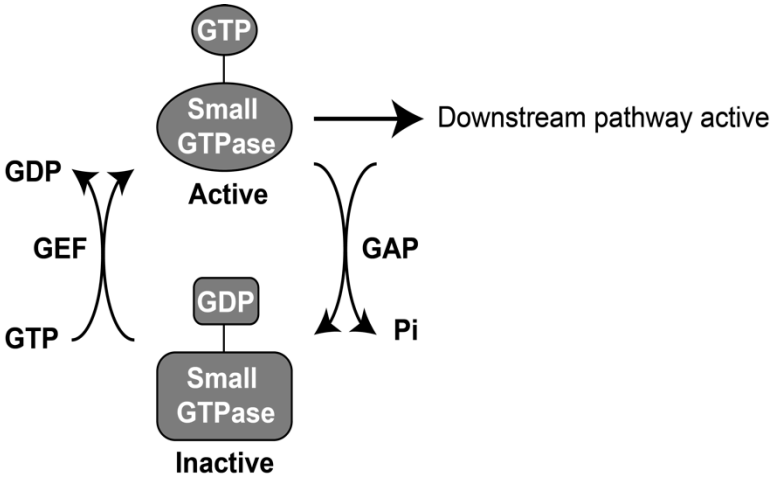

Figure 4: The GTPase activity cycle between the inactive GDP-bound state and the active GTP-bound state. The inactive state is promoted by GTPase activating proteins (GAPs) stimulating GTP hydrolysis, the active state is promoted by guanine nucleotide exchange factors (GEFs) loading GTP and dissociating GDP. The actived GTPase interacts with downstream effectors that in turn activate downstream signaling pathways. (Based on Nielsen et al., Plant Physiology, Aug 2008, 147 (4) 1516-1526)

1.2.2 The PAK family

p21-activated kinases (PAKs) is the best characterized downstream effectors of Rho family small GTPases[36,37]. PAKs are serine/threonine protein kinases with six isoforms. According to sequence similarity, PAKs can be divided into two groups, group I (PAK1-3) and group II (PAK4-6). PAKs contain p21-binding domain (PBD) at the N-terminus and a kinase domain at the C-terminus. Group I and II PAKs have different structures, therefore regulate their activity in different manners[38] (Figure 5). Group I PAKs form a dimer of two PAK molecules. The autoinhibitor domain (AID) partly overlaps with the PBD and binds to the kinase domain of another PAK molecule. When an activated small GTPase binds to PBD, this triggers AID disconnection from the kinase domain thereby releasing the kinase activity[38,39]. Group II PAKs is constitutively active and form monomers. Recent study shows they have autoinhibitory pseudosubstrate, which is similar to AID and adjacent to PBD. But the exact regulation is still unclear. There have two models available. Model 1: Activated

small GTPase bind to PAKs PBD, which lead to the kinase domain release. Model 2: Activated small GTPase bind to PAKs PBD, which trigers PAKs relocalization, and then the SH3 domain proteins bind to autoinhibitory pseudosubstrate, finally active the kinase activity[36,38].

As a downstream effector of Rho family small GTPases, the studies of PAKs initially focus on the cytoskeleton dynamics[40]. Later, PAKs have been shown to be involved in cancer progression, development of neuronal disease, viral pathogenesis, immunity and vascular disorders. Especially PAK1and PAK4 have been found overexpressed and amplified in many different cancer types, such as breast, pancreas, lung, ovary, prostate, gastric, leukemia, oral squamous-cell carcinoma and melanoma[36,41].

PAK regulates many cellular functions related to cancer progression, including cell proliferation, survival, cell morphology, adhesion and migration.

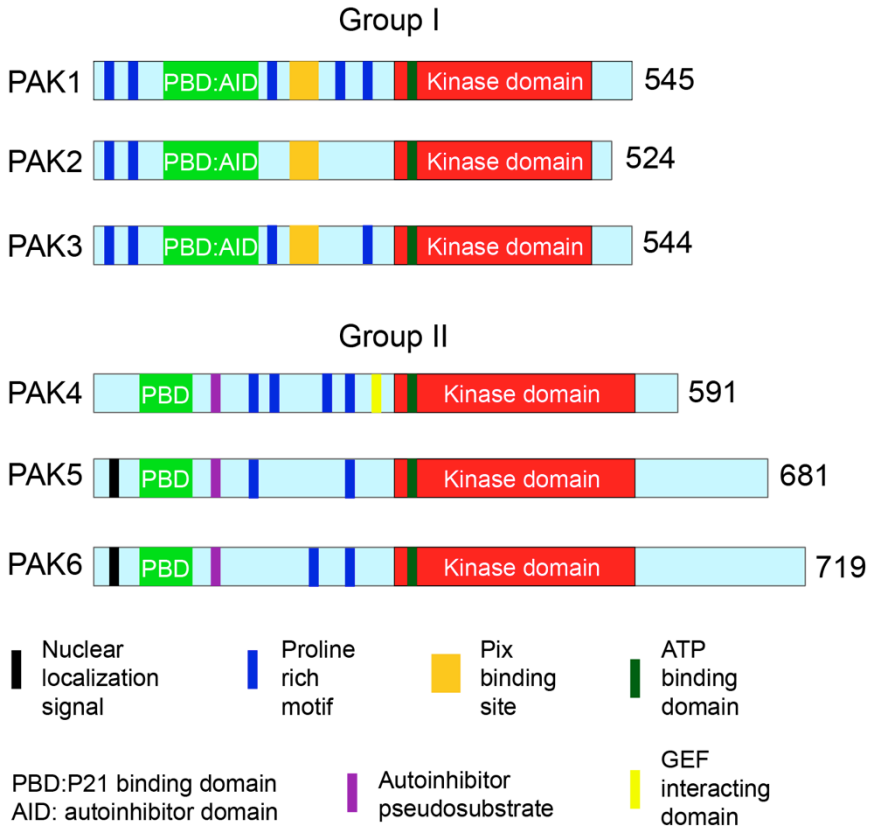

Figure 5: Domain structure of PAKs. All six PAKs have a serine/threonine kinase domain in the C-terminus and a PBD domain in the N-terminus. In all PAKs, an autoinhibition mechanism has been found. In group I PAKs, AID is overlap with PBD; In the Group II PAKs, an autoinhibitory psuedosubstrate was showed. The central region is more diverse. (Based on EYT al et., Journal of Molecular Signaling. 2014;9: 7.)

1.2.3 PAK4 function in cancer

PAK4 has been shown overexpressed or genetically amplified in lots of cancer cell lines and cancer types including breast, pancreatic, prostate, liver, gastric, ovarian cancer and oral squamous cell carcinoma[42-59]. Overexpression of PAK4 correlates with tumor aggressiveness,

metastasis[44,51,52] and poor prognosis[42,48,51]. Here I will focus on breast cancer and pancreas cancer. In MCF10A series of breast cell lines (MCF10A, MCF10AT, MCF10DCIS.com and MCF10CA1a), PAK4 expression is higher in more malignant MCF10CA1a[60]. PAK4 overexpression disrupts immortalized mouse mammary epithelial cells formed spherical acini with hollow lumen three dimensional architecture and normal apical/basolateral structures[61]. Another study shows that PAK4 is not just overexpressed in the breast cancer cell lines, but also overexpressed in breast cancer tumors, moreover, overexpression of PAK4 in NIH3T3 cells leads to tumor formation in athymic mice, while PAK4 deletion inhibit tumorigenesis[62]. More importantly, high PAK4 expression correlate with larger tumor size, lymph node metastasis, poor overall and disease-free survival[42].

PAK4 is also overexpressed and amplified in pancreatic cell lines and a fraction of pancreatic PDAC cancer patients[58,63,64]. Combining glaucarubinone and gemcitabine down-regulate active PAK4 in PANC-1 and MiaPaCa-2 cancer cell line, as well as in PANC-1 and MiaPaCa-2 xenograft, and then further reduce pancreatic cancer growth[65]. Knockdown of PAK4 restores sensitivity to gemcitabine in Capan-2, PANC-1, and SNU-410 pancreatic cell lines[66]. Data show PAK4 knockdown in a pancreas cancer cell line decrease cell cycle progression and apoptosis-resistance and also suppress growth and clonogenic ability[63].

1.2.4 PAK4 function in tissue development

PAK4 is ubiquitously expressed; its expression is high in early mouse development and low when mice are adults[67]. PAK4 conventional knockout (KO) mice display lethality by embryonic day 11.5, links to defects in the nervous systems, vessels and the heart. PAK4 KO embryos have thin neuroepithelia in the hind and fore brain, and therefore defects in neuronal differentiation and axonal outgrowth. Moreover, PAK4 KO embryos show fetal blood vessels together with maternal blood vessels in order to get proper nutrient delivery; Further, PAK4 KO embryos also display thin myocardial walls of the bulbous cortis and ventricle, which impairs ventricular function and cause its distended appearance[67].

Due to the early embryonic lethality of PAK4 KO mice, conditional PAK4 KO mice have been developed to study the role of PAK4 in different tissues. Epiblast-specific deletion of PAK4 mice still displays embryonic lethality and extraembryonic abnormalities[68]. Nervous system-specific deletion of PAK4 mice are born normal but exhibit growth retardation and die prematurely[69]. The secondary heart field conditional deletion of PAK4 shows abnormalities in the outflow tract and enlargement of the right ventricles and right atria[70]. However, the potential role of PAK4 in the pancreas has remained unknown.

1.2.5 PAK4 signaling

PAK4 is involved in cell cycle progression[63,71-73]. The expression level of PAK4 peaks in the early G1 phase and PAK4 knockdown decrease the amount of cells in the G1 phase and increase the amount of cell in the G2/M phase, which suggest that PAK4 plays an important role in cell cycle transition[72]. PAK4 may impact cell mitosis by two different aspects. Firstly,

PAK4 phosphorylate Ran on serine-135 and impedes its binding to RCC1 and RanGAP1 during mitosis[74]. Secondly, PAK4 is important for spindle positioning during mitosis[71,75]. PAK4 also regulates cancer cell proliferation by regulating several key players in the cell cycle, such as CDC25A, cyclin D1, CDK6, P16, and Smad2/3[63,76-78]. Further PAK4 may also regulate the PI3K /AKT pathway to achieve cell proliferation[42,55,63]. Taken together, all the evidences strongly suggest PAK4 significantly contribute to the cell proliferation.

Resistance to apoptosis is an important sign of oncogenesis. PAK4 promotes cell survival not only by increasing cell proliferation, but also inhibits apoptosis. To this end, PAK4 phosphorylates BAD, thereby blocking the interaction between BAD and BCL-XL and BCL-2 to prevent apoptosis[79]. Further, in response to certain stimuli, PAK4 abolishes Caspase 8 activation, thereby promoting resistance to apoptosis[80].

A role for PAK4 in transcription and translation has been indicated by several studies[81-83]. PAK4 associates with translational machinery components ribonucleoprotein complexes[83] and PAK4 also binds to the translation elongation factor Eef1a1[81] to regulate the HIF-1alpha translation in cancer cells[82].

1.2.6 PAK4 function in cytoskeleton dynamics and cell migration

The actin cytoskeleton is a dynamic structure that plays critical roles in several different cellular processes, such as cell morphology, cell adhesion and migration[84]. Actin cytoskeleton dynamics in cells are regulated by a large number of actin binding proteins. For example, formins promote new unbranched actin filaments; The actin-related protein 2/3 complex (Arp2/3 complex) promote nucleation of new branching actin filaments; ADF/cofilin proteins enhance actin filament severing and depolymerization; and also profilin and VASP proteins regulate actin filament polymerization[84].

Regulation of cytoskeletal organization, cell morphology, adhesion and migration are relatively well-characterized functions of PAK4. PAK4 induces filopodia formation by interaction with activated Cdc42 in Golgi[85]. PAK4 also changes the cell morphology by interaction with and phosphorylation of GEF-H1[86]. Moreover, PAK4 interacts with and phosphorylates LIMK1 and the slingshot homologue SSH-1, both part of a multi-protein complex with 14-3-3ζ, where PAK4 activates LIMK1, which in turn inactivates SSH1, leading to an increased ADF/cofilin activity that severs actin filaments and increases actin turnover[57,87-91]. PAK4 plays a key role in cell adhesion dynamics. Activated PAK4 expression induce focal adhesion disassembly either by phosphorylation of paxillin at Ser 272[56], or via phosphorylation of integrin β5, moreover, PAK4 promote integrin αvβ5 mediated cell migration by increasing integrin αvβ5 turnover within adhesions thereby destabilizing focal adhesion[92-94].

2 Aim of studies

The general aim of this thesis is to investigate the function of PAK4 in cells; tissue development and cancer progression, to better understand the role of PAK4 in cancer.

Special Aims:

Paper I: To elucidate the role of PAK4 in pancreas development.

Paper II: To investigate the PAK4 interactome and uncover new functions of PAK4.

Paper III: To determine the role of PAK4 in cellular senescence and breast cancer progression.

3 Methodological considerations

All the materials and methods used in this thesis are presented in detail in the papers, while general methodological considerations and limitations are discussed in this section.

3.1 Cancer Cell lines

Cancer cell lines derive from cancer patient are commonly used tools to study the biology of cancer and test the anti-cancer drugs, because they are cheap, infinite and easy to manipulate. However, cell cultures also have lots of limitations. 1) Lack of many tissue features: like blood vessels, oxygen pressure, hormone, original tissue structure and communication with different cell types. 2) Many number of passages may change characteristics, which no longer reflects the tumor from which it was derived[95,96]. 3) Different cell line cross-contamination[95].

Because normal cell culture has such limitations, we need better model to bridge the gap from bench to bedside. The three dimensional cancer cell cultures show better in studying dynamic interaction between cancer cells and surrounding extracellular matrix[97]. *Ex vivo* models, such as primary xenografts and orthotopic grafting, are more promising because these cancer cells have not adjusted to two dimensional tissue culture and are grown in a microenvironment that more closely mimics the context that they are derived from[98,99]. *In vivo*, cancer genetically engineered mouse models recapitulate many features of human cancer[100].

3.2 Genetically engineered mice

Genetically engineered mice are the most commonly used mouse models to study tissue development and cancer progression. Transgenic, conventional and conditional gene knockin and KO are common strategies to generate genetically engineered mice. To get pancreas-specific mouse models, cytokeratin 19, nestin, elastase, Mist1, p48 and Pdx1 have be used to limit the target gene expression to the pancreas[101]. In the paper I, we use the Pdx1 as the promoter to drive the Cre expression. Pdx1 starts to express from embryonic day 8.5, so it is a biomarker for pancreatic progenitor cells. Pdx1 expressed pancreatic progenitors later become adult ductal cells acinar and endocrine cells[101]. PAK4 protein expression level was below the detection limit in PAK4 KO mice using whole pancreas lysate shows Pdx1 promoter is good choice for the whole pancreas KO.

Genetically engineered mice have significantly contributed to our understanding of breast cancer initiation and progression. Breast cancer genetically engineered mice models are divided to different groups based on their features, including loss of tumor suppressor genes: p53, Brca1, Pten; or gain of an oncogene: Erbb2, Myc, H-ras and polyomavirus middle T (PyMT). To get mammary gland-specific mouse models, mammary tumor virus (MMTV), keratin-14 and whey acidic protein promoters have been utilized. In Paper III, we use MMTV

as a promoter, because most breast cancers originate from mammary gland epithelial cells and the MMTV promoter is mainly expressed in these epithelial cells. We employed an intensively studied breast cancer model driven by the MMTV-PyMT. We chose this model for two reasons: 1) This mouse model develops metastasis in lung and lymph nodes, while other transgenic models need to be combined to develop malignant cancers[102]. 2) This model shares many morphological and molecular features with human breast cancer. However, an obvious disadvantage of this mouse model is that PyMT itself is not expressed in human breast cancer[103].

3.3 Immunoprecipitation and mass spectrometry

Most proteins cannot execute their functions alone, but need to interact with other proteins to form macromolecular complexes to fulfill their functions. Combining immunoprecipitation (IP) and mass spectrometry (MS) is a rapid, sensitive, and reliable technique to study protein-protein interactions globally. There are three important steps for successfully identifying interactors using MS-based proteomics: 1) IP of the aim protein, 2) purification of the protein complex and 3) identification of the interacting proteins[104]. For the first step, since we have not been able to find a suitable antibody against endogenous PAK4, we used the FLAG antibody conjugated agarose beads to IP FLAG tagged PAK4 from cells stably expressing FLAG-PAK4. For the second step, since agarose beads can bring down many unspecific proteins[105], in order to get a purified protein complex, we used a FLAG peptide to elute the FLAG tag protein. For the third step, traditionally, after the purification of the protein complex, the sample will be injected to one dimension or two dimensions SDS-PAGE gel, finally compared the two lines or two gels to find the different bands or spots to identified using mass spectrometry[104]. However, recently developed isobaric tag for relative and absolute quantitation (iTRAQ)[106] labeled mass spectrometry (8-plex iTRAQ) gave us the opportunity to quantitatively analyze our IP eluates. iTRAQ also facilitated the simultaneous analysis of four repeats (4 controls and 4 samples), allowing statistical analysis and increasing the confidence of our data (Paper II; Figure 1).

Notably, proteins of the ribosome, spliceosome and ribonucleosome were enriched in the PAK4 interactome. These are potential candidates for false positive hits[105], yet PAK4 was also shown to play a role in translation[81,82,107]. We therefore have chosen to report these as well as all other hits passing our set cutoff.

4 Results and discussion

4.1 Paper I

Pdx1-Cre-driven conditional gene depletion suggests PAK4 as dispensable for mouse pancreas development

Conventional gene depletion of PAK1 or PAK3 caused functional deficits in the mouse pancreas[108,109], while gene depletion of PAK5 or PAK6 did not[110-113]. The knowledge of the potential role of PAK4 in pancreas development is lacking, so here we explored aforementioned problem. This study suggests that, like PAK5 and PAK6, PAK4 is dispensable for mouse pancreas development.

To generate conditional PAK4 KO mice targeting the pancreas, Pdx1-Cre mice and PAK4F/F mice were crossed[68,114]. Cre and PAK4 genotypes showed that mice with four different genotypes were born at the expected Mendelian ratio. Within the same genotypes, the numbers of male and female was approximately equal. The results suggest that deletion of PAK4 does not influence survival in any of the sexes.

Next, Haematoxylin and Eosin stainings revealed, similar to wild type (WT), acinar structures of PAK4 KO pancreas were evenly distributed and acinar cell cytoplasm was equal in strength. No obvious difference in ductal structures and in islet between two cohorts was observed.

To examine potential differences in islets between PAK4 KO and WT mice, area fraction covered by islets were measured and number of islets per pancreatic area were quantified, results displayed similarly for both genotypes.

The proportion and localization of cell types are critical for the pancreas to conduct its functions[115]. Amylase staining showed similar expression level in acini throughout the entire pancreas; CK19 stained ductal structures revealed a branched ducts system in the whole pancreas; Insulin staining β-cells formed the majority of the islets, while glucagon staining α-cells localized at the periphery of the islets with no differences between WT and PAK4 KO mice. Quantitative analyses of the islets also showed no significant differences were observed between WT and PAK4 KO mice.

Glucagon and insulin are major hormones for body weight regulation[116,117]. We here tested whether conditional PAK4 gene depletion may affect the body weight at different ages. Analysis of both female and male mice at one, two, four and six months of age showed an expected increase in body weight with age, but with no discernible differences between the two genotypes.

One of the important roles of the endocrine pancreas is maintenance of blood glucose

homeostasis[118]. To investigate whether loss of PAK4 may influence pancreatic function, we performed a glucose tolerance test and found WT and PAK4 KO mice displayed similar glucose induction and clearance curves, indicating that PAK4 gene depletion in the pancreas at two months does not affect the glucose regulatory function. Taken together, PAK4 is dispensable for mouse pancreas development.

4.2 Paper II

Identification of the PAK4 interactome reveals PAK4 phosphorylation of N-WASP and promotion of Arp2/3-dependent actin polymerization

PAK4 regulates cell motility, F-actin remodeling, cell proliferation and apoptosis [37,56,85,87,92,93,119-121], but the PAK4 interactome has not been systematically analyzed. In this paper, we comprehensively characterized the human PAK4 interactome by iTRAQ quantitative mass spectrometry of PAK4 - immunoprecipitations and found a new role for PAK4 in regulating the actin cytoskeleton.

Combining cell fractionation and iTRAQ based MS; we characterized 313 proteins as PAK4 interactors, 233 from whole cell lysates, 167 in the cytoplasmic fraction; and 54 in the nuclear fraction. By combined analysis of whole cell extracts and the two subcellular fractions (cytoplasmic and nuclear) enhanced the number of identified PAK4-associated proteins by an additional 80 hits.

We validated the previously known PAK4 interactors 14-3-3 α/β and 14-3-3 ε[122,123] and found a novel PAK4 interactor Arp2/3 complex subunit ARPC2. Then we identified actin cytoskeleton, 14-3-3, the replication fork and the proteasome these clusters enriched within the PAK4 interactome. Interestingly, the chaperonin containing TCP-1 complex (CCT complex) and the Arp2/3 complex, which previously not recognized to be associated with PAK4, were enriched in the actin cytoskeleton cluster. Further, we validate EGFP-PAK4 could co-immunoprecipitate ARPC2 as well as CCTε and anti-CCTε also could co-immunoprecipitate EGFP-PAK4.

Because PAK1 phosphorylate ARPC1B[124], we asked if PAK4 might also phosphorylate it. However, we did not detect any phosphorylation of the Arp2/3 complex subunits. Interestingly, we found that PAK4 phosphorylated the WASP VCA domain and Ser484/Ser485 in the VCA domain.

Further, we examined the association within PAK4, the Arp2/3 complex and the VCA domain. We found that the interaction between PAK4 and the VCA domain is direct and the association detected by co-IP between PAK4 and the Arp2/3 complex might be indirect. We also found endogenous PAK4 co-immunoprecipitated endogenous N-WASP and endogenous N-WASP co-immunoprecipitated endogenous PAK4. Importantly, N-WASP and PAK4 co-localized at F-actin bundles and leading edges at the cell periphery of MCF7 cells re-plated onto collagen type I.

Further study displayed that N-WASP Ser484/Ser485 phosphorylation decreased upon absence of PAK4 in H1299 cell. *In vitro* actin polymerization assay showed that pre-incubation of the VCA domain with the PAK4 kinase domain increased actin polymerization and G- and F-actin fractionation experiment displayed that knockdown of PAK4 did not change the total amount of actin, the balance between G- and F-actin was shifted towards G-actin. Finally, Immunofluorescence staining exhibited that PAK4 knock-down cells appeared more round with the phalloidin labeling enriched at the cell periphery, where the F-actin formed a cortical actin ring, while the control cells predominantly displayed an irregular shape and formed filopodia. Together, our results suggest that PAK4 dependent phosphorylation of N-WASP might promote actin polymerization, which is crucial for the cellular F-actin organization.

4.3 Paper III

PAK4 controls the non-canonical NF-κB component RelB to prevent senescence-like growth arrest in breast cancer

PAK4 is frequently overexpressed in many human cancers and contributes to multiple hallmarks of cancer[36]. However, the functional role of PAK4 in tumourigenesis *in vivo* remains largely unknown and the signaling pathways controlled by PAK4 in cancer remain poorly understood. This study uncovers a vulnerability of cancer cells to PAK4 inhibition that may be explored as a therapeutic strategy.

To examine expression levels of PAK4 and the potential prognostic ability for PAK4 in breast cancer patients in large cohort of breast cancer patients, we analyzed the METABRIC [125] and The Cancer Genome Atlas[126] data set and found that the level of PAK4 transcript was approximately two fold higher in breast tumors compared to their normal counterparts. We next asked weather PAK4 expression associated with the clinical outcome of breast cancer patients in the METABRIC cohort. The result showed that higher PAK4 expression was associated with worse disease specific survival and increased rates of mortality.

Using a genetic mouse model of PAK4 depletion to study tumourigenesis has not yet been employed, therefore we created conditional PAK4 gene depletion in PyMT transgenic breast cancer mouse model. We found elevated PAK4 protein levels in PyMT-driven mammary tumours. At 12 weeks of age, PyMT; PAK4 KO glands were less hyperplasic, exhibited fewer and smaller foci intermingled with normal ductal structures, and developed palpable tumours significantly later than PyMT; WT. Loss of PAK4 extended the median lifespan by 35 days in female and 152 days in male. Taken together, these observations indicate that mammary epithelial disruption of PAK4 results in impaired PyMT-induced tumourigenesis and thus that PAK4 contributes to PyMT mammary tumourigenesis.

The Gene Set Enrichment Analysis of RNA-Sequencing in Hs578T and BT549 revealed that senescence-associated gene signatures were overrepresented upon PAK4 depletion. Then we validate it *in vitro*, *in vivo* and *ex vivo* models. Upon siRNA-mediated PAK4 depletion,

Hs578T breast cancer cells adopted typical of a senescence response: flated and enlarged morphology, elevated enzymatic activity of SA-β-gal, decrease in BrdU-incorporation and upregulated the expression of genes involved in DNA damage/repair, cell cycle arrest and senescence associated secretory phenotype. We also examined the effect of PAK4 abrogation in a diverse collection of breast cancer cell lines, a primary cell line derived from a PyMT-driven tumour, tumour cells derived from breast cancer patients as well as cancer cells more diverse histological origins. Remarkably, most of them displayed increased SA-b-gal activity. Taken together, these findings show that inhibition of PAK4 triggers a senescence-like response in cancer cells of various histological origins.

We then expanded our analyses to additional *in vivo* models. We used two additional mouse models to test the relevance of PAK4 inhibition *in vivo*. Firstly, MCF-7 cells stably expressing shPAK4 or shControl were xenografted onto the back of nude mice. shPAK4 tumours grew slower and were smaller at the experimental endpoint than the tumours in the shControl cohort. Importantly, the tumours expressing PAK4 shRNA exhibited several hallmarks of senescence including abundant SA-β-gal activity, decreased Ki67-positivity and accumulated p53. To further test the *in vivo* function of PAK4 we treated the PyMT mouse model with the PAK4 inhibitor PF-03758309. Tissues were stained with X-gal and analyses revealed that PF-03758309-treated tumours had a higher proportion of X-gal-covered areas compared to DMSO-treated samples. Taken together, these experiments extend our findings to *in vivo* settings.

Further exploring our RNA-seq identified enrichment of NF-κB signatures upon PAK4 knockdown. In addition, we found a signature of NF-κB signaling strongly anti-correlated with PAK4 expression in the METABRIC breast cancer patient dataset and RelB, one of NF-κB subunit, had significant inverse association PAK4. Importantly, absence of RelB restored the proliferative capacity of Hs578T cells from the PAK4 knockdown-induced proliferation arrest showed that RelB is essential for growth arrest upon PAK4 depletion.

Furthermore, we found PAK4 physiological interacted RelB and phosphorylated the Rel-homology domain of RelB at serine 151 (S151). We also showed that the RelB S151E mutant had defective DNA binding and mimicking RelB-S151 phosphorylation decreased two major senescence regulators, Hes1 and CEBPB, mRNA expression. These results indicate that PAK4 is a RelB-kinase and that PAK4-dependent RelB phosphorylation on S151 is a novel regulatory mechanism that plays a critical role in the regulation of NF-κB signaling and senescence-associated factors.

5 Conclusions and future perspectives

In paper I, the pancreas specific PAK4 depletion mice have been examined and there was no obviously difference in morphology and functions compared to the WT mice. This suggests that PAK4 is dispensable for mouse pancreas development. This will facilitate future use of Pdx1-Cre-driven conditional PAK4 KO mouse model for testing potential *in vivo* functions of PAK4 in pancreatic disease models such as for pancreatitis and different pancreatic cancer forms. Previous studies indicates that PAK4 is overexpressed and amplified in a fraction of pancreas adenomcarcinoma cell lines as well as in corresponding patient material. However, until now, there are no papers using a transgenic mouse model to study the function of PAK4 in pancreas cancer progression. Crossing PAK4 KO model with pancreas cancer models, such as the KC model (Pdx1-Cre; LSL-Kras $^{G12D/+}$) or KPC Model (Pdx1-Cre; LSL-Kras $^{G12D/+}$; LSL-p53$^{R172H/+}$) model, will possibly elucidate the role of PAK4 in pancreas cancer progression.

In paper II, we comprehensively characterized the human PAK4 interactome, which provides a valuable resource for future investigations on the role of PAK4 in physiology and disease. The PAK4 interactome provides a substantial number of new leads for future studies of the roles and molecular functions of PAK4. In this study, we found several key proteins of the DNA replication fork in the PAK4 interactome, suggesting that PAK4 may regulate the DNA replication fork, adding complexity to the role of PAK4 in cell proliferation. Another potential function of PAK4 interacting proteins is to control PAK4 activity and stability. The ubiquitin-proteasome system and the lysosomes are common ways to degrade proteins. We found a large number of proteasome subunits in the PAK4 interactome, which is consistent with the recent report that PAK4 can be ubiquitinated and degraded by the proteasome.

We found that PAK4 regulates the actin organization by interacting with and phosphorylating N-WASP at Ser484/Ser485. We thereby propose a new mechanism for PAK4 regulation of actin cytoskeleton dynamics. However, at this stage, it remains elusive how PAK4 may help balance the interplay between actin polymerization and depolymerization at the cell leading edge to achieve rapid cell migration, which will require further investigations. In addition, we report a novel interaction between PAK4 and the CCT complex, a chaperonin that is essential for folding of the cytoskeletal proteins actin and tubulin and. Several CCT subunits have also been shown to regulate the actin cytoskeleton and the formation of cell surface protrusions. However, whether the CCT complex is a PAK4 substrate and if their association may be involved in regulation of the actin cytoskeleton remains to be examined.

In paper III, we establish PAK4 as a protumourigenic regulator of breast cancer acting through the non-canonical NF-κB subunit RelB. Our study thus uncovers a vulnerability of cancer cells to PAK4 inhibition that may be explored as a therapeutic strategy.

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